GENETIC FINGERPRINTS AND PHYLOGENETIC RELATIONSHIPS OF EIGHTEEN APHID SPECIES FROM EGYPT (HEMIPTERA: STERNORRHYNCHA: APHIDIDAE)

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ABSTRACT: The current study aimed to identify eighteen aphid species belonging to Tribes Macrosiphini and Aphidini (Subtribe: Rhapalosiphina) by using Biochemical and Molecular genetic markers (isozymes, SDS-Page total protein and RAPD-PCR markers) as well as surveying of biochemical and RAPD–PCR species – specific bands for some of those tested species. Each of isozyme and RAPD electrophoresis analysis revealed the highest level of polymorphism, comparing with total protein electrophoresis analysis. The electrophoresis study for those different molecular systems revealed that 160 different bands pattern, seven of them were considered as common bands in all tested species, while thirty four bands were observed in some species as species - specific bands. The electrophoresis studies in those different molecular systems reflected 95.21% polymorphism among the tested species. Phylogenetic relationship based on combined effect of isozyme, Total protein and RAPD-PCR analysis reflected that the highest similarity was recorded between *Rhopalosipum maidis* and *R. padi*, dendrogram analysis can separate the Greaminaceae host plant aphid from the other species. Moreover it showed clearly the gab between Tribe: Macrosiphini and Tribe: Aphidini (Subtribe: Rhapalosiphina). A molecular branching key was constructed to identify thirteen species out of the eighteen tested species. This key is depended on species-specific markers.

KEY WORDS: Genetic fingerprints, Phylogentic relationships, Aphids, Molecular branching key.

Family Aphididae comprises more than 4400 aphid species placed in 493 genera, which from one of the most prolific groups of insects. Because they are capable of not only rapid increase of population though parthenogenesis but also transmission of plant viral diseases and secreting honey dew which become suitable media for sooty moulds. So they are regarding as one of the most important groups of agricultural pests. In addition aphid attack many economic important host plants such as horticultural, forest trees and field crops (Minks & Harrewijn, 1989). This family includes eight Sub-families; Aphidinae is considered as the largest sub families contains most widespread aphid in Egypt; many species have host alternation between woody and herbaceous angiosperms; few species live on Conferee and ferns, the majority on higher angiosperms (Blackman & Eastop, 1984).

Aphids are considered as polymorphic species. Species identification mostly depends on the morphological features of the alate adults. Morphological characters alone have proven inadequate in differentiation between closely related species, which is known to be greatly influenced by either environmental factors such as climatic conditions or the physiological status of their host plants. Moreover, various morphs of the same species can occur during the same time

interval, making aphid identification in some cases a very difficult and conflicted task. In addition, closely related taxa, which often cannot be distinguished morphologically, colonize different host plants (Guldemond, 1991). In the past, variation of biological properties between clones of the same species had also been described. The host plants were used for species or subspecies differentiation (Blackman, 1987). Chromosome measurements were also used to separate closely and related species and biotypes of aphid species (Mayo et al., 1988), which was incapable to solve some taxonomic problems.

On the other hand, although biochemical techniques such as allozymes electrophoresis have been developed and showed better diagnosis for some aphid species, it haven't been useful tool to indicate variability which has often been detected in the biological attributes of various aphid species (May & Holbrook, 1978). Also, molecular markers may be helpful in solving this problem (Yeh-Hsin et al., 2005). A diverse range of novel molecular (DNA) markers are now available for entomological investigations. Both DNA and protein markers have revolutionized the biological sciences and have enhanced many fields of studies. Relative to DNA markers, alloenzymes are cheap, often much quicker to isolate and develop, even from minute insects *i.e.* aphid (Aphidoidea), thrips (Thysanoptera), parasitic wasps (Hymenoptera), etc., and subsequently easy to use (Loxdale & Lushai, 1998).

These modern techniques are now available for systematic studies of aphid. Mitochondrial DNA analysis was used to differentiate between closely related aphid species (Footit & Bonen, 1990), biotypes (Powers et al., 1989) and clones (Martinez et al., 1992). Also using of random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) has proven useful for detection and differentiation of the wide range of organisms. Moreover it can be useful mean in studying closely related species, (Black et al., 1992 and Puterka et al., 1993).

So the current study continues to complete aphid biochemical and molecular genetic fingerprints with other aphid species. The study concentrated on common eighteen aphid species belonging to Tribe Macrosiphini and Tribe Aphidini (Sub-tribe Rhapalosiphina), to detect species – specific molecular marker, which can be used to construct a molecular key used in identification procedure with some tested aphid species, moreover to study phylogenetic relationship depending on these parameters.

MATERIAL AND METHODS

Samples Collection

Some aphid species belonging to Tribe: Macrosiphini and Sub-tribe: Rhapalosiphina were surveyed and collected from main host plants in some localities of Egypt, through out two successive years extended from December, 2005 to June, 2007. Surveyed species are listed alphabetically with scientific names in Table (1). Alate forms of each species were preserved in 70% ethyl alcohol and stored till specimen mounting.

Specimen (alate and apterus forms) of each species were collected from their host plants and put in glass jar covered with muslin cloth and transferred to the laboratory. Data about host plant, date of collection, locality and color of each specimen were recorded. In laboratory, five clones of each species with apart of host plant were preserved in glass jar covered with muslin under dark condition till alate forms appear. Mounted specimens were identified by using professional taxonomic keys of aphids such as Habib & El-Kady (1961) and Blackman & Eastop (1984, 2000) to identify species.

One adult apterus female from each identified species was reared on its fresh and healthy host plants and caged separately by leaf cages under laboratory conditions, its offsprings of the second generation for each stem mother were collected and preserved in Eppendorf tubes at –20 °C until subjected to biochemical genetic analyses (Isozyme and SDS-PAGE) and DNA fingerprint analysis (RAPD- PCR).

Biochemical Genetic Characterizations

Isozyme Electrophoresis.

Aphid species were subjected to Native – polyacrylamide gel electrophoresis (Native- PAGE) to identify isozyme variation among them. So in this study, four enzymatic systems (α- Esterase, β- Esterase, Acid phosphatase and Alkaline phosphatase) were extracted from adult apterus females gel according to Stegmann et al. (1985) and separated in 9% Polyacrylamide. Then gel stained according to Scadalios (1964) and Wendel & Weeden (1989).

SDS Protein Electrophoresis.

The eighteen aphid tested species were subjected to Sodium Dodecyl Sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to study the total protein profiles of these species for appreciation in species discrimination. Mini – Gel Electrophoresis apparatus (Bio-Rad), supplemented with vertical slab (8.5 cm x 9cm) were used for this purpose.

Protein extraction buffer was prepared according to Hames & Rickwood (1981). Hundred µl of extraction buffer were added to the Eppendorf's contains ten adult apterus female homogenized together. Extraction was left overnight in -20 °C then vortexed for 15 second, centrifuged at 12.000 rpm at 4 °C for 15 minutes, transferred to new Eppendorf tubes and kept under -20 °C till using in protein electrophoresis.

Molecular Genetic Characterizations

The Eighteen tested aphid species were subjected to Random Amplified Polymorphic DNA – Polymerase Chain Reaction (RAPD- PCR) with ten arbitrary ten-mer primers, which synthesized by Operon Biotechnology. Inc. Germany. The primer sequences are as follow:-

Primer Code	Nucleotide Sequence 5\ ---------- 3\
B_{10}	CTGCTGGGAC
C_{15}	GACGGATCAG
D_2	GGACCCAACC
D_5	TGA GCGGACA
I_{17}	GGTGGTGATG
L_{12}	GGGGGGTACT
L_{13}	ACCGCCTGCT
L_{20}	TGGTGGACCA
Z_1	TCTGTGCCAC
UBC $_{75}$	GAGGTCCCAA

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L_{13}	ACCGCCTGCT
L_{20}	TGGTGGACCA
Z_1	TCTGTGCCAC
UBC $_{75}$	GAGGTCCCAA

DNA Extraction:

Five individuals of apterus aphid were grinded in 1.5 Eppendorf tube with 200 µl of cooled extraction buffer (1.5 gm NaCl, 2.42 gm Tris – HCl, 0. 5 gm SDS, 0.73 gm Na_2- EDTA, 100 ml distillated water, adjusted to pH 8) in liquid nitrogen, then 100 µl of Sodium acetate (3M) were added and vortexed gently. The tubes were placed under–20 ^oC for 15 minutes then centrifuged at 10.000 rpm at 4 ^oC for 5 minutes. The supernatants were transferred to a new Eppendorf tube with equal volume of cold Isopropanol, then vortexed gently and placed under – 20 ^oC for 1 hour. The precipitated DNA was pelleted by centrifuged at 13.000 rpm at 4 ^oC for 20 minutes. The supernatant was poured off gently and 100 µl of Ethanol 70% were added and centrifuged at 10.000 rpm at 4 ^oC for 5 minutes, then Ethanol poured off gently and the tubes were inverted on filter paper for 30 minutes, which DNA pellets appear on the bottom and was left to dry. Finally, the pellets were dissolved in 100 µl of Tris -EDTA buffer (TE) (0.151 gm Tris – base, 0.029 gm Na_2- EDTA, 100 ml distillated water adjusted to pH 8.) and added 2.5 µl of RNAse.

Polymerase chain reaction (PCR) preparations and conditions:

Amplification reaction was performed with 20 µl final volume of reaction mixture [10 µl Prime Taq™ Premix (2X), 2 µl Primer, 7 µl Injection water, 1 µl Genomic DNA (25 ng)].

Amplification was performed in a thermal cycler (Perkin Elmer Gene Amp. PCR System 2400) with Initial Denaturation (Initial strands separation) on 94 ^oC for 4 min., then 35 cycles each cycle contained Denaturation on 94 ^oC for 45 Sec., Annealing on 37 ^oC for 45 Sec. and extension on 72 ^oC for 1.5 min. finally, the program was ended with final extension 72 ^oC for 10 min.

The PCR products were electropherized in 1.2% Agarose gel at 110 volts by using Bio – Rad DNA electrophoresis system. Then gel analysis was carried out by using Bio-Rad Quantity one computer system version 4.0.3.

Phylogenetic Relationships:

Each of bands variations in Isozyme, SDS-PAGE and RAPD-PCR were subjected individually to statistical analysis. Then those parameters were also pooled together to it, to study the Phylogenetic relationship among the eighteen aphid species on each level and to generate dendrogram by using SPSS computer program version 13 and applying Dice equation.

RESULTS AND DISSCUSION

Biochemical Genetic Characterizations

Isozyme Electrophoresis.

Electrophoretic profile of α-Esterase isozyme for the eighteen tested aphid species are presented in Figure (1a). These results revealed that α-Esterase gave high levels of variations that give seven polymorphic α-Esterase isozyme bands. Both *Myzus persicae* and *Metopolophium dirhodum* gave the highest number of banding pattern (7 bands); in contrast, *Acyrthosiphon pisum* gave the lowest banding pattern (1 band). The α-Esterase 4 (α-est4) was with frequency in most tested aphid species.

Results of β-Esterase isozyme pattern are illustrated Figure (1b), which reflected that the eighteen aphid species gave eight β-Esterase isozyme with polymorphism variation 87.50 %. β-Esterase 1 (β-est1) was common band in all tested aphid species, while β-est5, β-est7 and β-est8 were unique bands for *Rhopalosiphum maidis, Macrosiphum rosae* and *Brevicoryne brassicae*, respectively.

Results of electrophoresis pattern of acid phosphatase for the eighteen tested aphid species are illustrated in Figure (1c). This enzyme gave eight isozymes in tested species with polymorphism 100%. Acph1 was the most frequent isozyme band in tested species except in *Myzus persicae*. Each of Acph3, Acph7 and Acph8 were unique bands for *Brachycaudus schwartzi*, *Capitophorus elaeagina* and *Nasonovia (Hyperomyzus) lactucae*, respectively.

Electrophoretic profile of alkaline phosphatase isozymes is illustrated in Figure (1d). These results revealed that four isozymes of alkaline phosphatase were found in the tested aphid species, with polymorphism 100%. Aph1 was the most frequent isozyme in the tested species except *Brachcaudus helichrysi*, *Chomaphis inculata*, *Myzus persicae* and *Hyalopterus pruni*. Aph4 was unique band for *Hyalopterus pruni*. Alkaline phosphatase isozymes were totally absent in each of *Brachcaudus helichrysi, Chomaphis inculata* and *Myzus persicae*.

Esterases enzymes gave a diagnostic and specific banding patterns, can separate *Sitobion avenae, Rhopalosiphum padi* and *R. maidis*. (Singh & Cunningham, 1981). Also, Esterase banding pattern most useful tool to separate the different genera as well as individual species of *Sitobion avenae*, *S. fragariae*, *Metopolophium dirhodum*, *M. festucae*, *Rhopalosiphum padi* and *R. maidis* (Loxdale et al., 1983). *Schizaphis graminum* gave nine Esterase bands, the 5th and 6th bands were stained darker (Sivakumaran & Mayo, 1991). *Myzus antirrhinii* was distinguished electrophoretically from both *M. persicae* and *M. nicotianae* by its distinctive patterns of Esterases (Blackman & Spence, 1992). Esterase isoenzyme analysis can be used to differentiate between *Myzus persicae* and *Rhopalosiphum padi* (Vranac et al., 1994). Nine bands were found for Esterase in *Sitobion avenae, Rhopalosiphum padi* and *Schizaphis graminum* (Celis et al., 1996). In addition, electrophoratic banding pattern of α- Esterase and β- Esterase for ten aphis species, collected from Egypt, gave seven and eight bands,

respectively. α-est5 was species specific band for *Aphis nerii*, and α-est2 was common band; while β-est1 and β-est7 were expressed in *A. compositae* and *A. medicaginis*, respectively, and β-est2 was common band (Amin et al., 2008).

Acid and Alkaline phosphatase electrophoresis could be used to discriminate each of *Sitobion avenae*, *Rhopalosiphum padi* and *R. maidis* (Singh & Cunningham, 1981). Also among *Sitobion avenae*, *S. fragariae*, *Metopolophium dirhodum*, *M. festucae*, *Rhopalosiphum padi* and *R. maidis* (Loxdale et al., 1983) among *Sitobion fragariae, Macrosiphum funestum* and *Amphorophora rubi* (Loxdale & Brookes, 1989). Electrophoratic banding pattern of Acid phosphatase and Alkaline phosphatase for ten aphis species, collected from Egypt, gave four bands. Acph1 appeared as specific for *Aphis compositae*; while Aph1 and Aph2 were expressed as unique bands in each of *A. citricola* and *A. compositae*, respectively. Aph bands pattern were absent in each of *A. medicacaginis, A. rumicis, A. zizyphi, A. gossypii* and *A. faba*. It was suggested that Ach isozyme could discriminate among the tested *Aphis* species (Amin et al., 2008).

SDS- Protein Electrophoresis.

Total protein electrophoresis pattern among the eighteen aphid species is illustrated in Figures (2). Thirty two protein bands were recorded along SDS-Polyacrlamide gel. Molecular weights of recorded bands ranged from 14.366 to 245.578 KDa. Two monomorphic bands were observed in electrophorasis with molecular weights of 100.654 and 25.929 KDa, respectively. On the other hand, one of protein band with molecular weight of 23.789 KDa was recorded as unique band in *Capitophorus elaeagina*. The most frequent polymorphic band was 126.379 KDa, which observed in most species except in each of *Macrosiphum (Sitobion) avenae* and *Metopolophium dirhodum*. These results showed that total protein electrophoresis harbored 93.75% polymorphism among the eighteen tested aphid species, which gave adequate level to discriminate among them, because of they are belong to different species, genera and moreover different tribes.

Protein assay was not able to distinguishing *Myzus persicae* clones collected from different locatities (Baker, 1979). *Aphis pomi* gave two protein loci (Singh & Rhomberg, 1984). *Uroleucon gobonis, Aphis craccivora* and *Greenidea formosana heeri* exhibited 11, 13 and 26 protein bands, respectively (Khuda-Buksh & Khuda-Buksh, 1991). Moreover, protein electrophoresis didn't differentiate among *A. gossypii* population in Japan (Owsus et al., 1996). Total protein electrophoresis for ten *Aphis* species collected from Egypt, gave twenty three bands with molecular weights ranged from 13.49 to 118.23 KDa. Protein band with molecular weight 13.49 KDa was recorded as unique band for *Aphis punicae*. (Amin et al., 2008).

The present work showed that 25.929 KDa is common band in the eighteen tested aphid species may be the same common band (25.63 KDa), recorded by Amin et al. (2008) with the ten *Aphis* species.

Molecular Genetic Characterizations:

RAPD-PCR analysis.

The RAPD analysis as shown in Figure (3) with the ten primers gave 101 different DNA fragment bands with wide molecular sizes. Four monomorphic distinct fragment bands were recorded; most of them were occurred with primers B10 and L13. So, the lowest value of polymorphism (80%) was generated by them.

On the other hand, the highest polymorphic bands were produced by primers C15, D2, I17, L12, L20, UBC75 and Z1 to achieve polymorphism levels reaching 100%. The highest number of DNA fragment bands (fifteen bands) was observed in primer D5, while the lowest number was five bands, generated by primer L13. Twenty six DNA fragment bands were expressed as species – specific bands. Five of them occurred in primer I17, while primer L12 gave one species- specific band. The complied data for the ten primers recorded 95% polymorphism among the eighteen tested species.

Primer B10 produced ten DNA fragment with the eighteen tested aphid species with molecular size ranged from 246 bp to 2402 bp. Each of *Capitophorus elaeagina* and *Pentalonia nigronervosa* gave one unique band with molecular weight 900 and 830 bp, respectively.

The generated RAPD profile of DNA fragment bands with the primer C15 gave the highest number of bands (7 bands) in each of *Doctynotus sonchi* and *Hyalopterus pruni*. Each of *Brevicoryne brassicae* and *Doctynotus sonchi* gave unique band with molecular sizes 1282 and 1497 bp, respectively.

Two unique bands with molecular size 1132 and 171 bp were noticed and recorded as specific band for *Brevicoryne brassicae* and *Schizaphis graminum* species, respectively with primer D2. *Schizaphis graminum* harbored seven DNA fragment bands to record the highest number of bands with this primer. In contrary, *Brachcaudus helichrysi* didn't give any banding pattern with this primer.

Primer D5 produced fifteen DNA fragment bands with wide molecular size extended from 129 to 1967 bp. One monomorphic DNA band was recorded in all tested species in molecular size 181 bp to reveal polymorphism degree reaching to 93.33%. On the other hand, each of *Capitophorus elaeagina*, *Doctynotus sonchi* and *Hyalopterus pruni* had species- specific bands with molecular sizes 129, 1198 and 1401 bp, respectively.

DNA fragment bands profile with arbitrary primer I17 gave fourteen DNA bands, which were polymorphic and unique bands that could adequate to help in discrimination procedure of the eighteen aphid tested species. Five bands are recorded as species – specific bands that observed with *Brevicoryne brassicae*, *Macrosiphum (Sitobion) avenae*, *Hyalopterus pruni*, *Schizaphis graminum* and *Nasonovia (Hyperomyzus) lactucae* in molecular sizes 1171, 1555, 759, 565 and 326 bp, respectively.

It was obvious from bands analysis that the PCR products generated by L12 primer were eight bands with molecular sizes ranged from 191 to 2843 bp with 100% polymorphism among the eighteen tested species. One unique band with molecular size 237 bp was detected as species – specific band for *Rhopalosiphum maidis*.

Primer L13 gave limited number of DNA fragment bands (five bands) with molecular sizes ranged from 227 to 1146 bp, and reflected 80% polymorphism among tested species. One common band was detected in all species in molecular size 1146 bp. On the other hand, three unique bands were noticed; two of them with molecular sizes 227 and 420 bp can be species - specific bands for *Doctynotus sonchi*, in addition to another one with molecular sizes 303 bp was observed in *Brachcaudus helichrysi*.

The bands of DNA fragment electrophoresis with primer L20 are graphically illustrated eight bands with molecular sizes ranged from 163 to 2084 bp. One species – specific band was found in *Hyalopterus pruni* with molecular size 2084 bp. the lowest number of DNA bands pattern were found in *Doctynotus sonchi* (one band).

Primer UBC75 generated eight different DNA fragment bands with molecular sizes ranged from 201 to 1983 bp. *Chomaphis inculata* harbored the highest number of DNA bands with this primer (five bands), while the lowest number of band (one band) was found in *Macrosiphum (Sitobion) avenae*. Four unique DNA fragment bands were observed, two of them were species- specific bands in *Chomaphis inculata*, which their molecular sizes 1983 and 1366 bp. While the others were species – specific bands in each of *Pleotrichophorus chrysanthemi* and *Nasonovia (Hyperomyzus) lactucae* with molecular sizes 639 and 201 bp, respectively.

Primer Z1 produced thirteen DNA fragment bands with wide molecular sizes ranged from 143 to 3894 bp. Three different species – specific bands were detected with primer Z1 in each of *Capitophorus elaeagina*, *Rhopalosiphum padi* and *Brachcaudus helichrysi*, which their molecular sizes were 2250, 225 and 143 bp, respectively.

The RAPD-PCR analysis gave difference not only among different genera but also for differentiation between different species of the same genus, when used with *Rhopalosiphum padi, Aphis gossypii, A. fabae A. craccivora, Myzus persicae,* and *Acyrthosiphan pisum* (Cenis et al., 1993). It was suitable method to determine genetic distances among different taxa (families, subfamilies, genera, species and populations within species) of aphids, moreover for differentiation and identification of aphids especially for closely related species at DNA level (Zhang et al., 2000). RAPD –PCR analysis gave detectable genetic polymorphism between *Myzus persica* complex and *M. cerasi, M. hemerocallis* and *M. varians* species; in addition to, *Myzus persica* and *M. nicotianae* could be considered as synonyms (Clements et al., 2000). Each of geographical and seasonal distribution of *Sitobion avenae* populations had low effect on genetic variability (Figueroa et al., 2005). Each of primers B10, D2, I17, L12, L13 and Z1 generated species specific markers in six *Aphis* species (*A. craccivora, A. faba, A. nerii, A. punicae, A. rumicis* and *A. zizyphi*)*;* primer D2 was the most efficient primer, because of it could distinguish four *Aphis* species (*A. faba, A. nerii, A. punicae* and *A. rumicis*) (Amin et al., 2008).

Survey of Biochemical and RAPD–PCR Species – Specific Marker for the eighteen Tested Aphid Species:-

Thirty four diagnostic markers for the eighteen aphid species were surveyed and presented in Table (2), which based on biochemical genetic analysis (α-Esterase, β-Esterase, Acid phosphatase, Alkaline phosphatase isozymes and total protein) and RAPD – PCR analysis with the ten arbitrary primers.

Isozyme analysis gave seven species - specific markers except α- Esterase didn't gave any species- specific markers. Most of obtained isozyme markers were observed in each of β- Esterase and Acid phosphatase isozyme analysis. The isozyme analysis could distinguish only the following species (*Brevicoryne brassicae*, *Brachycaudus schwartzi*, *Capitophorus elaeagina*, *Nasonovia (Hyperomyzus) lactucae, Macrosiphum rosae*, *Rhopalosiphum padi* and *Hyalopterus pruni*). On the other hand, total protein analysis could distinguish only *Capitophorus elaeagina* by protein band with molecular weight 23.789 Kda.

Obtained results revealed that abundant numbers of DNA fragment species – specific marker were generated by the ten arbitrary primers comparing to isozyme and total protein electrophoresis analysis, which reveal the importance and effectiveness of RAPD – PCR analysis. It can distinguish thirteen aphid species out of the eighteen aphid species. Each of *Acyrthosiphon pisum*, *Brachycaudus schwartzi*, *Myzus persicae*, *Macrosiphum rosae* and *Metopolophium dirhodum*

didn't generate any species- specific markers with the ten used primers, so those five species should be tested with other random primers to detect their species – specific bands in further studies. In contract, *Doctynotus sonchi* harbored the highest number of DNA species – specific bands with the ten used primers. Primer I17 was the most effective primer which generated the highest number of species - specific marker. So it can distinguish five aphid species [*Brevicoryne brassicae*, *Nasonovia (Hyperomyzus) lactucae*, *Macrosiphum (Sitobion) avenae*, *Hyalopterus pruni* and *Schizaphis graminum*]. While each of primers L12 and L20 gave one species – specific marker, which can distinguish *Rhopalosiphum maidis* and *Hyalopterus pruni*, respectively.

From aforementioned results, it could be concluded that fifteen species out of eighteen could be differentiated well by applying electrophoresis analysis program based on only β- Esterase and Acid phosphatase isozyme analyses in addition to application of RAPD-PCR analysis with primers B10, I17, L12, L13, UBC75 and Z1 which can save time, material and efforts with the fifteen aphid differentiated species. So it scored all species – specific makers, which can differentiate among them.

Proposal Molecular Branching Key for Identification of Thirteen Aphid Species:

During the present work of molecular genetic characterizations by using ten random primers with the eighteen tested aphid species, 26 DNA species – specific bands were detected. Eighteen DNA species – specific bands out of the twenty six DNA species – specific bands, generated by main six primers (B10, I17, L12, L13, UBC75 and Z1) out of the ten random primers, were used successfully to construct a molecular branching key to identify thirteen aphid species out of the eighteen tested aphid species (Figure 4). While the other species - specific bands (8 bands), generated by the other primers (C15, D2, D5 and L20) were used only in this key as confirming bands for the thirteen species which can be used after identify those aphid species with the main six primers.

Phylogenetic Relationships:

Results of proximity matrix analysis for the eighteen aphid species based on isozyme polymorphism reflected that the highest similarity value 88.9% was recorded between *Chomaphis inculata* and *Doctynotus sonchi*, while the lowest similarity in isozyme polymorphism (25%) was recorded between *Brachcaudus helichrysi* and *Schizaphis graminum*. Moreover, dendrogram analysis based on isozyme polymorphism is graphically illustrated in Figure (5), which reflects that the eighteen tested aphid species can be classify into two main groups depending on isozyme polymorphism analysis. The first main group divided to two sub groups, the first includes *Chomaphis inculata*, *Doctynotus sonchi*, *Brachcaudus helichrysi*, *Capitophorus elaeagina*, *Pleotrichophorus chrysanthemi*, *Myzus persicae* and *Metopolophium dirhodum* while the second includes *Brachcaudus schwartzi* and *Hyalopterus pruni*. The second main group contains also two sub groups; the first one includes *Brevicoryne brassicae*, *Nasonovia (Hyperomyzus) lactucae*, *Acyrthosiphon pisum*, *Rhopalosiphum padi*, *Schizaphis graminum*, *Macrosiphum (Sitobion) avenae* and *Rhopalosiphum maidis*; while the second sub group includes *Pentalonia nigronervosa* and *Macrosiphum rosae*.

Results of the eighteen aphid species based on RAPD-PCR polymorphism analysis agree with proximity matrix analysis of total protein, which reflected that the highest similarity value was recorded between *Rhopalosiphum maidis* and *Rho. padi*, and each of them can also separate the Greaminaceae host plant aphid

from others. Moreover it showed the gab between Tribe Macrosiphini and Tribe Aphidini (Sub-tribe: Rhapalosiphina).

Total protein dendrogram analysis, graphically illustrated in Figure (6) showed that the eighteen tested aphid species can be classified into two main groups depending on total protein polymorphism analysis. The first main group divided into two sub groups, the first includes the Greaminaceae host plant aphid *i.e. Rhopalosiphum maidis*, *Rho. padi*, *Hyalopterus pruni*, *Schizaphis graminum*, *Macrosiphum (Sitobion) avenae* and *Metopolophium dirhodum* while the second includes *Acyrthosiphon pisum*, *Brevicoryne brassicae*, *Capitophorus elaeagina*, *Doctynotus sonchi*, *Brachycaudus schwartzi* and *Bra. helichrysi*. The second main group contains also two sub groups; the first one includes *Chomaphis inculata* and *Myzus persicae*; while the second sub group includes *Pleotrichophorus chrysanthemi*, *Macrosiphum rosa, Pentalonia nigronervosa* and *Nasonovia (Hyperomyzus) lactucae*.

Polymorphism dendrogram analysis of DNA is graphically illustrated in Figure (7). Results showed that the eighteen tested aphid species can be classify into two main groups depending on DNA polymorphism analysis. The first main group divided into three sub groups, the first includes the Greaminaceae host plant aphid [*Rhopalosiphum maidis*, *Rho. padi*, *Hyalopterus pruni*, *Schizaphis graminum*, *Metopolophium dirhodum* and *Macrosiphum (Sitobion) avenae*], in addition to *Pleotrichophorus chrysanthemi* and *Macrosiphum rosae*; The second sub group includes *Chomaphis inculata*, *Nasonovia (Hyperomyzus) lactucae*, *Myzus persicae*, *Acyrthosiphon pisum*, *Pentalonia nigronervosa*, *Brevicoryne brassicae* and *Capitophorus elaeagina*. The third sub group contains *Doctynotus sonchi* only. On the other hand, the second main group includes two closely related species *Brachycaudus helichrysi* and *Bra. schwartzi*.

Results of proximity matrix analysis for the eighteen aphid species based on combined effect of isozyme, Total protein and RAPD-PCR analysis reflected that the highest similarity value (89.9%) was recorded between *Rhopalosiphum maidis* and *Rho. padi*, Dendrogram analysis based on them is graphically illustrated in Figure (8), which can separate the Greaminaceae host plants aphid from other species. Moreover it showed clearly the gab between Tribe Macrosiphini and Tribe Aphidini (Sub-tribe: Rhapalosiphina). These studies declared that the eighteen tested aphid species can be classified into two main groups depending on combined effect of isozyme, Total protein and RAPD analysis. The first main group divided into three sub groups, the first sub group includes the Greaminaceae host plants aphid *i.e. Rhopalosiphum maidis*, *Rho. padi*, *Schizaphis graminum*, *Hyalopterus pruni*, *Macrosiphum (Sitobion) avenae* and *Metopolophium dirhodum*. While the second sub group includes *Acyrthosiphon pisum, Brevicoryne brassicae, Capitophorus elaeagina* and *Doctynotus sonchi*. The third sub group contains *Pleotrichophorus chrysanthemi*, *Macrosiphum rosae, Chomaphis inculata* and *Myzus persicae, Nasonovia (Hyperomyzus) lactucae* and *Pentalonia nigronervosa*. On the other hand, the second main group includes two closely related species *Brachycaudus helichrysi* and *Bra. schwartzi*.

Both of *Aphis gossypii* and *Myzus persicae* showed a higher genetic differentiation than *Rhopalosiphum padi* (Martinez et al., 1997). The genetic similarities coefficient ranged from 0.414 to 0.808 in two morphological types of *Myzus persicae* collected from tobacco plants. Moreover, no correlation between morphological types and RAPD polymorphism was detected (Chae-Soon et al., 1998). *Myzus persicae* on rape plants was more closely related to those from tobacco than those on peach (Yang-Xiao et al., 1999). There was a close

relationship between the green spruce aphid, *Elatobium abietinum* in two localities (Sigurdsson et al., 1999). Each of *Aphis citricolla* and *A. compositae* were the most closely related species depending on RAPD-PCR analysis or on combined effect of isozyme, Total protein and RAPD analysis, with similarities values 88.6% and 84.45%, respectively (Amin et al., 2008).

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Table 1. List of eighteen aphid species under consideration with their host plants and localities.

	No. of Aphid	Aphid species	Host plant	Locality
Tribe: Macrosiphini	1	*Acyrthosiphon pisum*	*Pisum sativum*, *Vicia Faba*	El- Behera, Qalyubiya, Giza
	2	*Brevicoryne brassicae*	*Brassica oleracea* *Brassica rapa*	Kafer El-Shakh, El- Behera, Qalyubiya
	3	*Brachcaudus helichrysi*	*Ageratum houstonianum*, *Myosotis sp.*, *Prunus armeniaca, P. persica*	Cairo El- Behera
	4	*Brachycaudus schwartzi*	*Prunus armeniaca*, *Prunus persica*	El- Behera
	5	*Capitophorus elaeagina*	*Cynara scolymus*	El- Behera
	6	*Chomaphis inculata*	*Foeniculum vulgar*	Kafer El-Shakh, El- Behera
	7	*Doctynotus sonchi*	*Sonchus oleraceus*	Qalyubiya, Giza
	8	*Myzus persicae*	*Solanum tuberosum, Lactuca sativa, Petunia hybrida Linaria bipartite*	Qalyubiya Cairo
	9	*Nasonovia (Hyperomyzus) lactucae*	*Sonchus oleraceus*	El- Behera Qalyubiya
	10	*Pentalonia nigronervosa*	*Musa sapientum*	El- Behera Qalyubiya
	11	*Pleotrichophorus chrysanthemi*	*Silybium marianum*, *Calendula officinalis*	Qalyubiya
	12	*Macrosiphum rosae*	*Rosa hybrida*	El- Behera Giza
	13	*Macrosiphum (Sitobion) avenae*	*Triticum aestivum*, , *Hordeum vulgare*, *Setaria glauca*	Kafer El-Shakh El- Behera Giza
	14	*Metopolophium dirhodum*	*Triticum aestivum*, *Hordeum vulgare* , *Avena fatua*	El- Behera Giza
Tribe:Aphidini	15	*Rhopalosipum maidis*	*Hordeum vulgare*, *Triticum aestivum*	El- Behera
	16	*Rhopalosipum padi*	*Triticum aestivum*, *Setaria glauca*	Kafer El-Shakh, El-Behera
	17	*Hyalopterus pruni*	*Aronda donax* , *Prunus armeniaca*	El-Behera
	18	*Schizaphis graminum*	*Sorghum spacchartum*	Beni-Suef El-Behera

Table 2. Summary of surveyed biochemical and RAPD-PCR species – specific marker for the eighteen tested aphid species.

Biochemical and Molecular Marker \ Aphid species		*Acy. pisum*	*Bre. brassicae*	*Bra. helichryst*	*Bra. schwartzi*	*Cap. elaeagina*	*Com. inculata*	*Doc. sonchi*	*Myz. persicae*	*Nas. (Hyp.) lactucae*	*Pen. nigronervosa*	*Ple. chrysanthemi*	*Mac. rosae*	*Mac. (Sit.) avenae*	*Met. dirhodum*	*Rho. maidis*	*Rho. padi*	*Hyl. pruni*	*Sch. graminum*
Enzyme (isoenzyme)	α - Esterase																		
	* β- Esterase		Est8										Est7				Est5		
	* Acid phosphatase				Acph3	Acph8				Acph7									
	Alkaline phosphatase																	Aph4	
Protein weight (KDa)	Total protein					23.789													
Code of Arbitrary primer (Molecular Size) (bp)	* B10					900					830								
	C15		1282					1497											
	D2		1132																171
	D5					129		1198										1401	
	* I17		1771							326				1555				759	565
	* L12															237			
	* L13			303				420 227											
	L20																	2084	
	* UBC75						1983 1366			201		693							
	* Z1			143		2250											225		

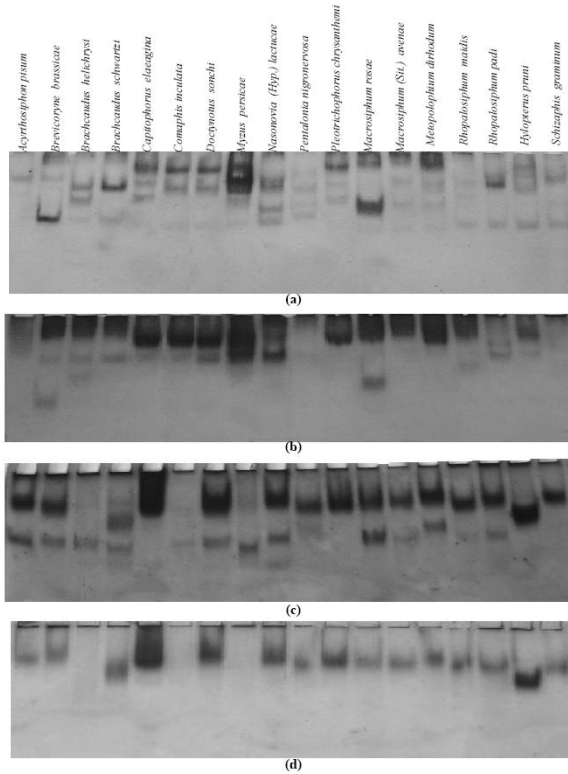


Figure 1. Zymogram of α- Esterase (a), β- Esterase (b), acid phosphatase (c) and alkaline phosphatase (d) banding pattern of the eighteen tested aphid species in Egypt.

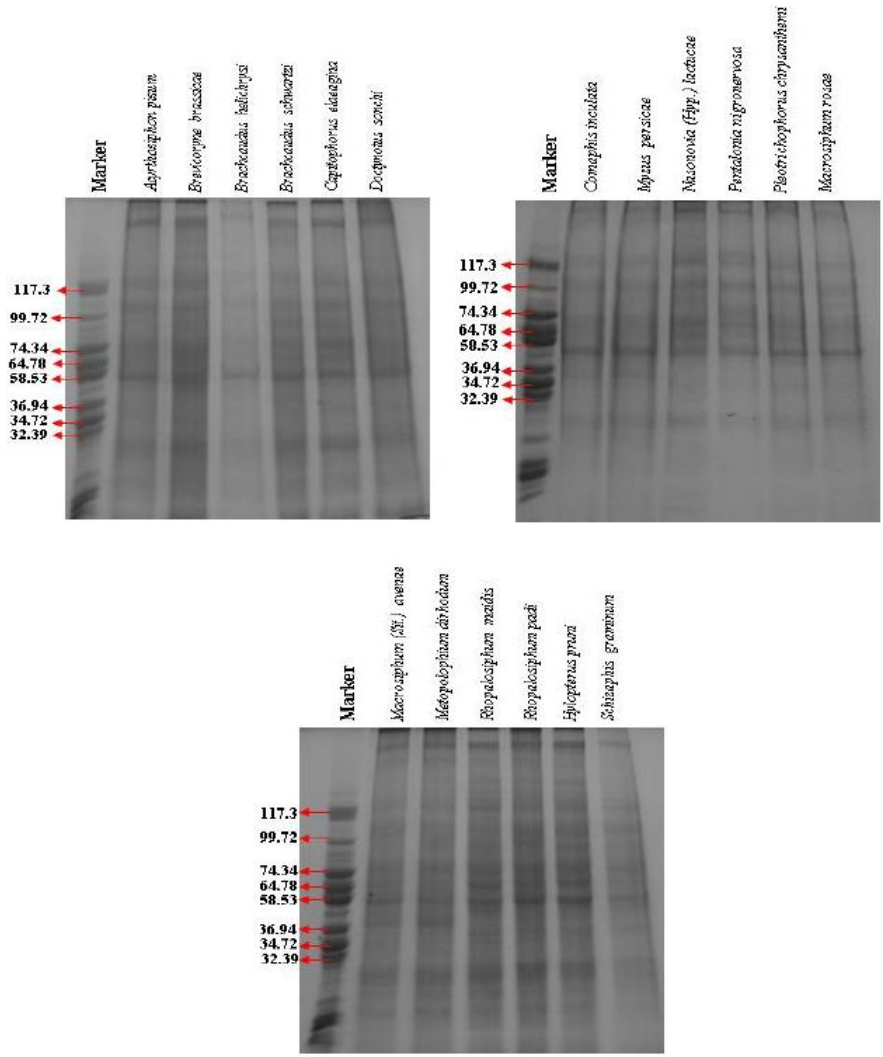
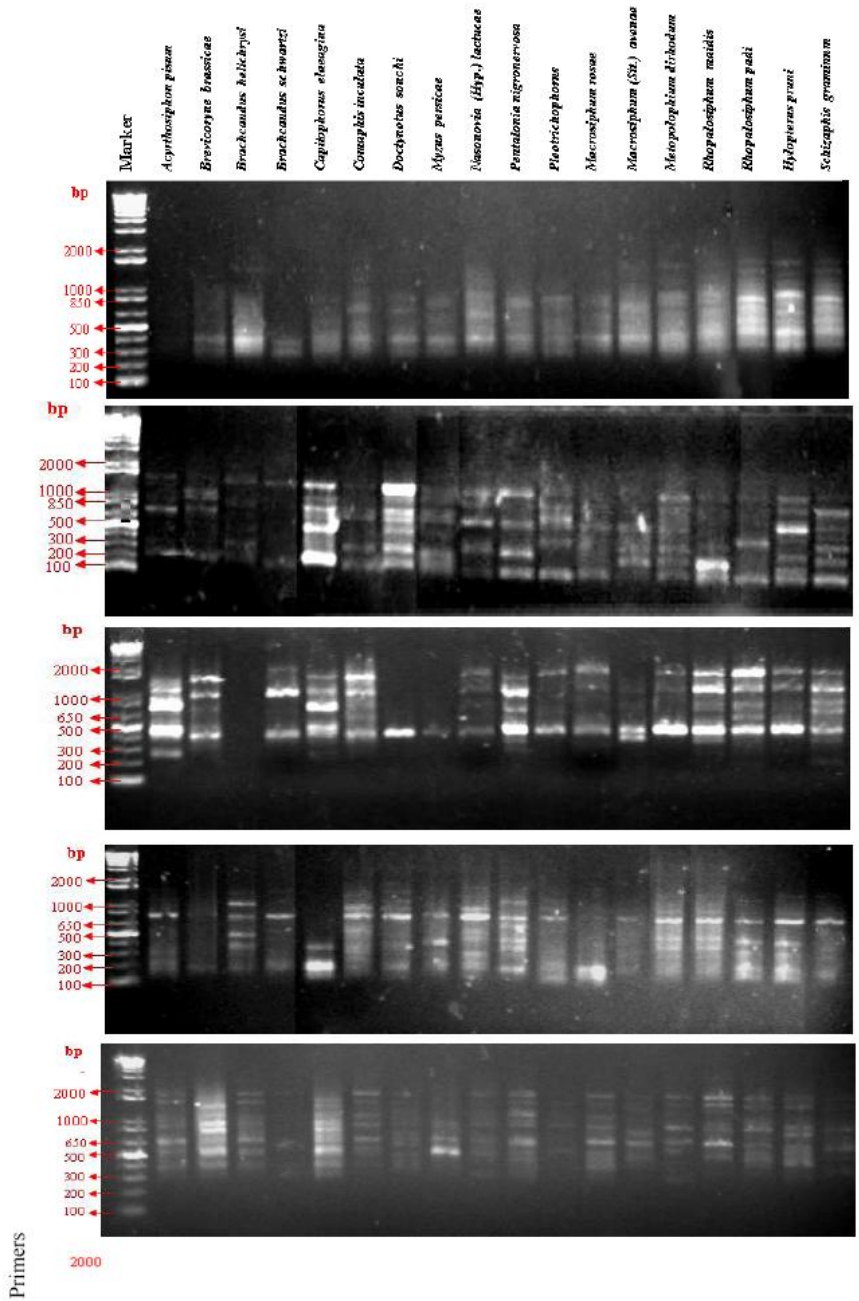


Figure 2. Zymogram of SDS- protein banding pattern for the eighteen tested aphid species.


Marker
Acyrthosiphon pisum
Brevicoryne brassicae
Brachcaudus helichrysi
Brachcaudus schwartzi
Capitophorus elaeagnus
Cavariella aegopodii
Dactynotus sonchi
Myzus persicae
Nasonovia (Hyp.) lactucae
Pentalonia nigronervosa
Plesticlinophorus
Macrosiphum rosae
Macrosiphum (Sit.) avenae
Metopolophium dirhodum
Rhopalosiphum maidis
Rhopalosiphum padi
Hyperomyzus pallidus
Schizaphis graminum
bp
2000
1000
850
650
500
300
200
100
Primers
2000

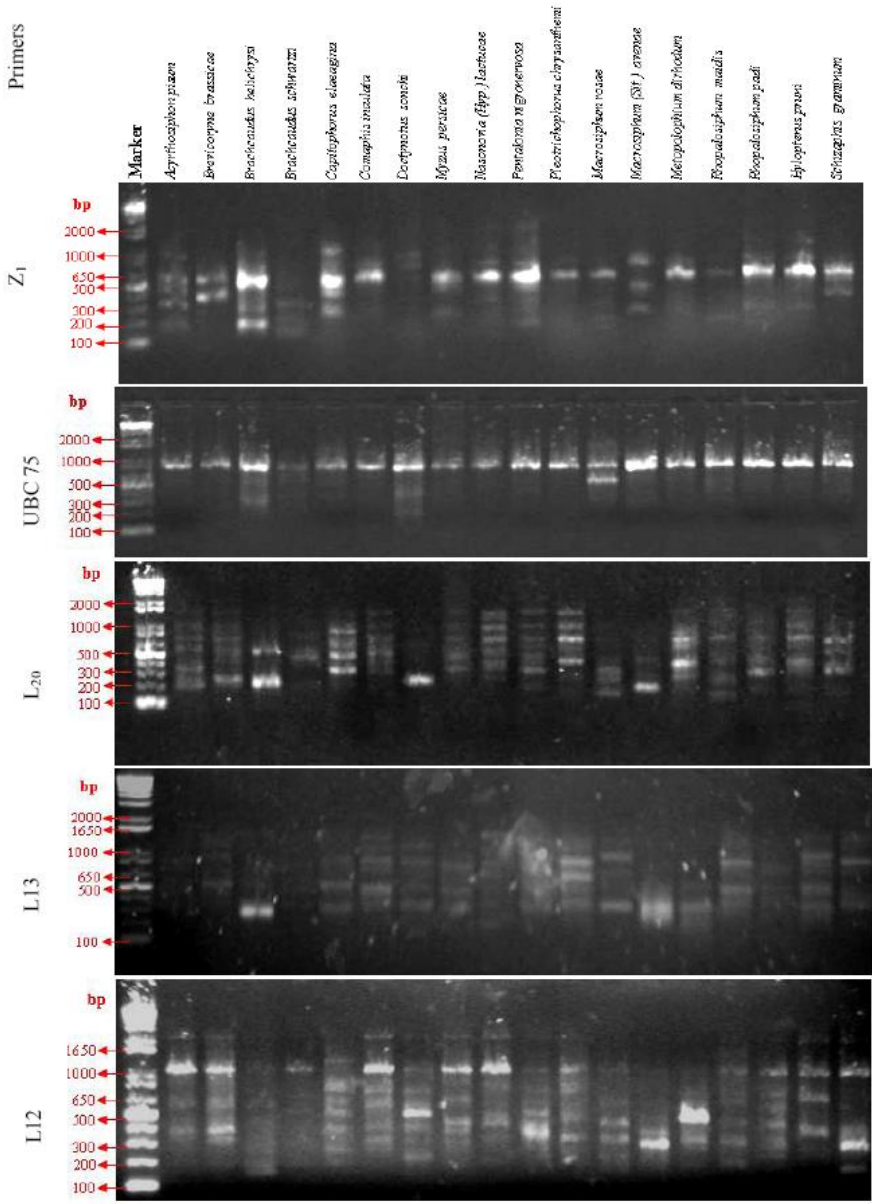


Figure 3. DNA fragment bands generated by ten arbitrary primers in the eighteen aphid species.

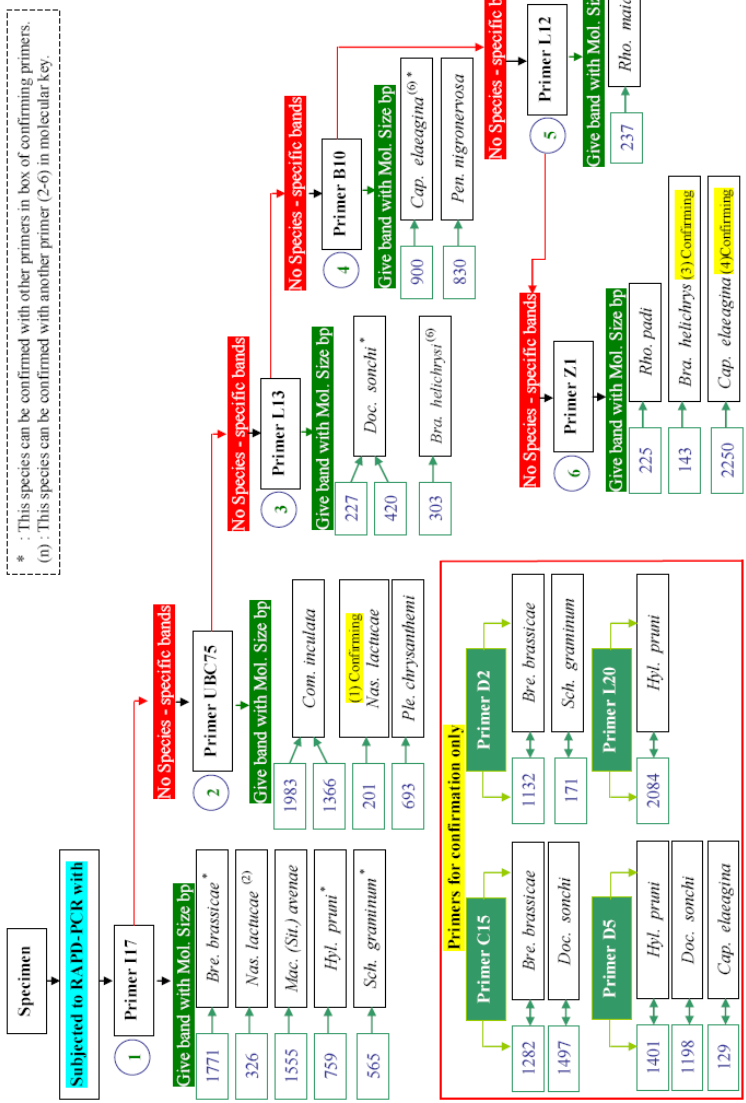


Figure (4): Branching molecular Key for identifying thirteen aphid species by using species –specific markers.

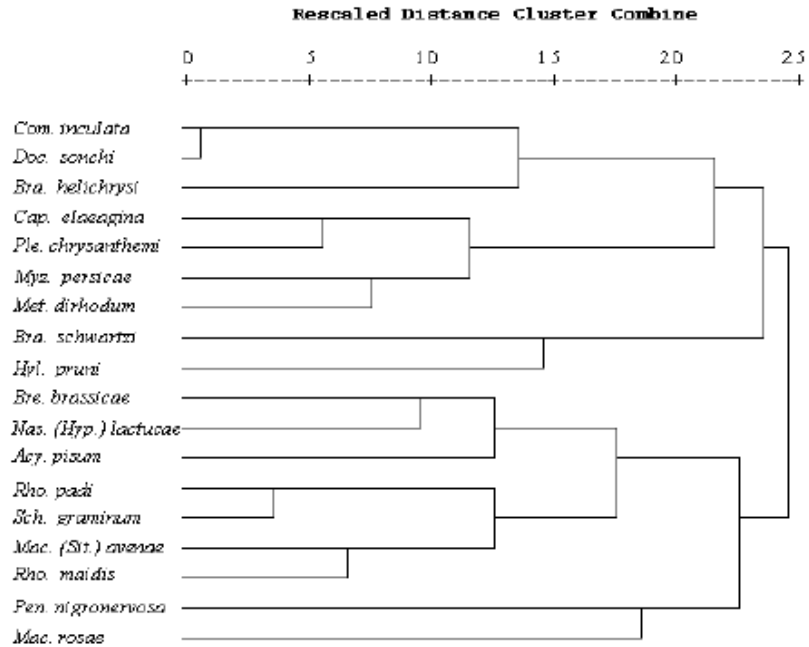


Figure 5. Dendrogram show phylogenetic relationship among the eighteen aphid species based on isozyme banding pattern analysis.

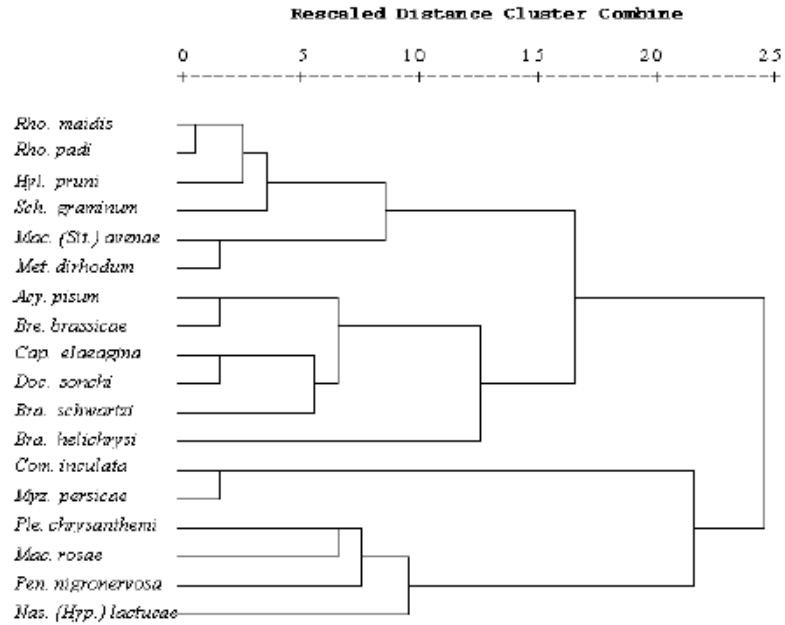


Figure 6. Dendrogram show phylogenetic relationship among the eighteen aphid species based on total protein banding pattern analysis.

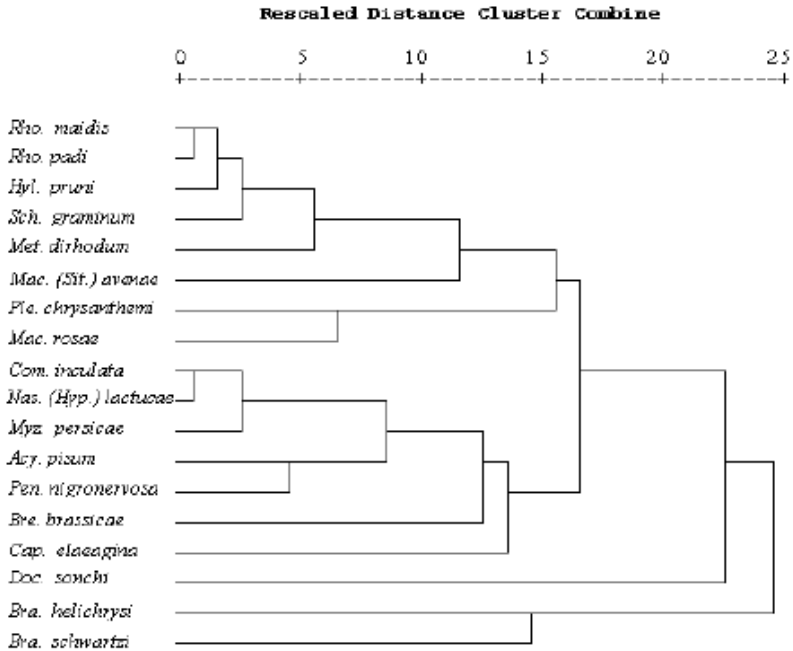


Figure 7. Dendrogram show phylogenetic relationship among the eighteen aphid species based on RAPD banding pattern analysis.

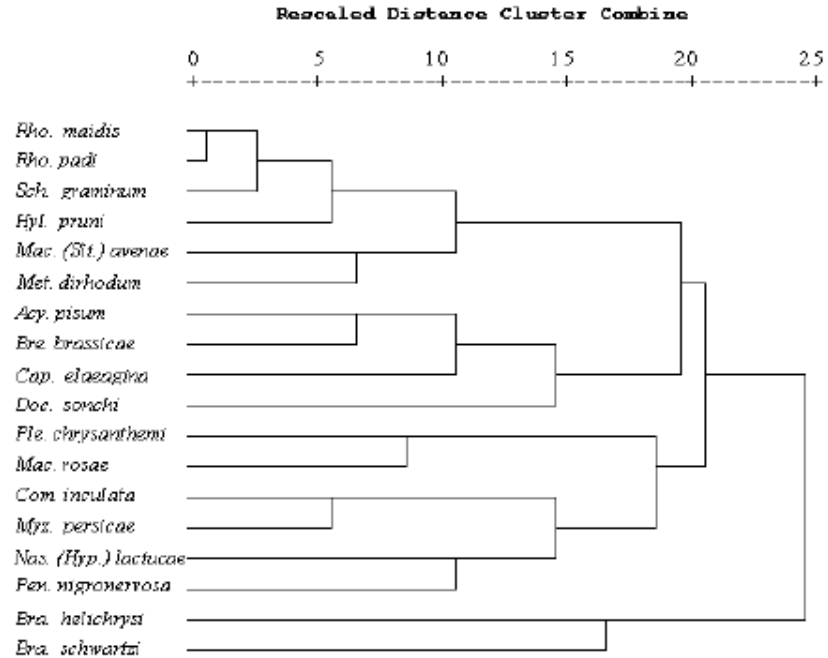


Figure 8. Dendrogram show phylogenetic relationship among the eighteen aphid species based on combined effect of isozyme, Total protein and RAPD analysis.